

## CRISPR-STAT (Somatic Tissue Activity Test) Protocol

Blake Carrington 8/12/15

### 1. Collection of Tissue (Embryos) for DNA extraction

Collect 8 uninjected (WT) and 8 injected embryos at 24-72hpf; 1 embryo/ well.

- Use 96-well PCR plates to collect tissue for DNA extraction, making sure to place embryo at the bottom of the wells for efficient DNA extraction. Care must be taken when transferring embryos to avoid excess water in the wells (up to 10ul of water will not affect DNA extraction).
- If embryos are in chorion place them in -20°C freezer for 20 minutes to break chorions to allow extraction solutions access to the embryo.

### 2. DNA Extraction

Our method is optimized to work with DNA extracted using the Extract-N-Amp kit (Sigma) as described below. However, DNA extracted by other methods also work equally well.

All steps are carried out at room temperature (RT) unless noted.

- Solutions in steps 1 and 2 can be premixed and 32ul can be added to sample.

1. Add 25ul of Extraction Solution to sample.
2. Add 7ul of Tissue Preparation Solution. Mix well but make sure tissue remains submerged in solution or extraction will fail. **The use of a vortex is NOT recommended here.**
3. Incubate at RT for 10 minutes.
4. Incubate samples at 95°C for 5 minutes.

Note: Tissue will not be completely dissolved at the end of the incubations. This is normal and will not affect performance.

5. Add 25ul of Neutralization Solution B to sample and mix by vortexing.
6. Store DNA at 4°C for up a week, -20°C for longer periods or use immediately for PCR.
7. Make 1:10 dilution (10ul DNA: 90ul H<sub>2</sub>O) of DNA to use for fluorescent PCR reaction. **Critical step** for this method of DNA extraction to remove PCR inhibitors from DNA for efficient amplification.

### 3. Setting up fluorescent PCR Reaction

1. Make "PCR Brew" (to make 5mL (~50 aliquots): This can be scaled up or down)

794ul	10x PCR Gold Buffer (contains no MgCl <sub>2</sub> )
794ul	25mM MgCl <sub>2</sub>
198.75ul	10mM dNTP mix
3.15ml	Ultra Pure H <sub>2</sub> O
63.5ul	Taq Gold

Aliquot 100ul of brew into tubes and store at -20°C

2. Make Primer mix (all primer stocks are prepared as 100uM)

Forward primer (M13F tailed)	5ul
Reverse primer (pig tailed)	5ul
M13F-FAM or M13F-HEX primer	5ul
TE pH8.0	485ul

3. Set up PCR reaction as follows (Note: If you are testing multiple targets that will be amplified with the same primer set you only need to run the WT control PCR for that primer set with 8 embryos.)

- a. Thaw 1 tube of PCR brew (100ul) and add 6ul of primer mix.
- b. Put 5ul of "brew + primer mix" into each well.
- c. Add 2ul of diluted DNA and place in thermocycler.

**PCR conditions:**

94°C for 12 min  
40 cycles of  
94°C for 30s  
57°C for 30s  
72°C for 30s  
72°C for 10 min  
4°C forever

**4. Fragment analysis of PCR products by capillary electrophoresis**

1. Prepare size standard dilution as 1:50 dilution of ROX400 size standard in HiDi formamide
2. Mix diluted size standard and PCR product as below:  
2 ul PCR product  
10ul ROX400/ HiDi mixture
3. Denature for 5 minutes at 95°C. **(CRITICAL STEP)**
4. Run on ABI capillary electrophoresis Genetic Analyzers, such as 3130xl or 3730xl.
5. Process .fsa files using GeneMapper or Genescan and Genotyper software packages (Applied Biosystems).

**5. Analyze data to determine if CRISPR has somatic activity.**

1. First look at your WT samples to make sure you are seeing 1 peak per sample. (If you have 2 peaks due to an endogenous indel that is ok but you need to take that into account when you are looking for secondary peaks in your injected samples.)
2. Look at your injected samples to determine if you have activity. See examples for how to "score" activity. This can be done by eye and does not need to be quantitated.

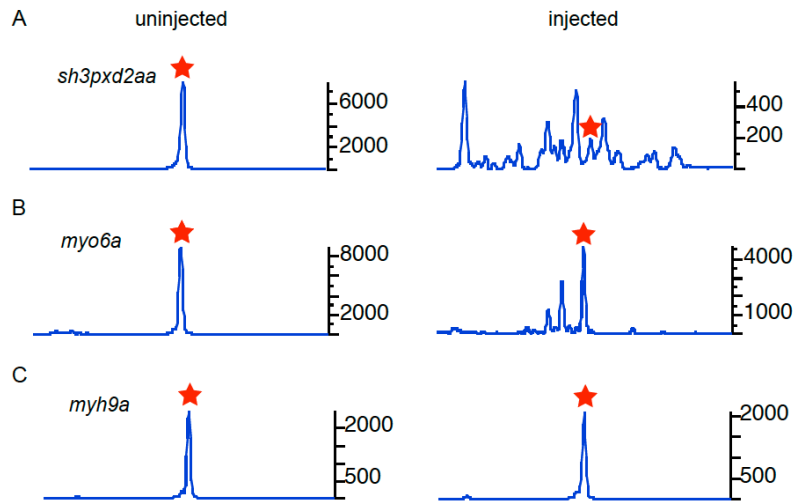
## Examples of activity:

Here we have 3 CRISPRs with different activity levels (A-high, B-low and C-none). When scoring your injected embryos you can use these plots as guidelines for your activity levels. Based on the activity level you should also be able to predict germline efficiency rates so you can predict the number of founders you will need to screen to recover germline mutations.

High activity >50% of founders should transmit mutations

Low activity <50% of founders should transmit mutations

No activity – very few to no founders will give you germline mutation



Uninjected embryo in left panel.

CRISPR injected embryo in right panel.

Red star denotes WT allele.

Gene	<i>sh3pxd2aa</i>
Target Sequence	GGTGCTGGAGCAGTACGTGG
For primer (w/ M13F tail)	tgtaaaacgacggccagtGTTACGCTAGGTTGCGGAGT
Rev primer (w/ PIG-tail)	gtgtctttTGTAAAGGACTCACCGCTTT
PCR product size	295 bp

Gene	<i>myo6a</i>
Target Sequence	GGATCAAGTCTTCCCTGCTG
For primer (w/ M13F tail)	tgtaaaacgacggccagtTGGGTTAAATGCAGAGCACA
Rev primer (w/ PIG-tail)	gtgtctttCACACACACGAACTCACG
PCR product size	262 bp

Gene	<i>myh9a</i>
Target Sequence	GGGTCGTGATTTTGTTTCTAGA
For primer (w/ M13F tail)	tgtaaaacgacggccagtGCTTTGTTGATGCGCATGAC
Rev primer (w/ PIG-tail)	gtgtctttTCCCTTTTATCAGCTGCCCA
PCR product size	237 bp

### Primer Design:

Since zebrafish are highly heterogeneous, it is best to test the target region for SNPs and in/dels by sequencing a few wildtype fish of the strain you plan to use for mutagenesis. Design primers to amplify 125 to 350bp product (include 25 nucleotides for primer tails) with the target site in the middle of the amplicon. An ideal amplicon size is between 200-300bp. Follow standard criteria for primer design: 18-21 nucleotides long with 40 to 60% GC content, at least 3 of the last 6 nucleotides as C or G and the 3' nucleotide to be a C or G.

To the 5' end of amplicon-specific primers, add tail sequences as below:

M13F: TGTAACGACGGCCAGT

Pigtail: GTGTCTT

### Example:

In this amplicon marked by yellow highlights for forward and reverse primers, green highlight marks the target site and blue highlights mark the translation start site and splice site.

AGAGTTAGTGGACGCAAAGTGTGTAGTGGAGAGAAGTGTGTTTAGTTAGTAAAGAAGCT  
GTAGCCATGCCCCCTAGCACACAAGAGGACGATACCGTCTCCGGTATACGGAAAGGCATA  
CGGGCCAATTCTGCTCGGGCCGCCGGCGGGGAAGGGAACGCAGGTGAGAGTTTATCCA  
AAGGGCCGCGTGGCTCATTCTGAAGCGGGATAGTGCTCTGGGTTATTGAGGTGAA

Primer sequences to be ordered are:

Forward primer:

TGTAACGACGGCCAGTAGAGTTAGTGGACGCAAAG

Reverse primer:

GTGTCTTTCACCTCAATAACCCAGAG

Product size: 234bp + 25bp = 259bp

In addition, order an M13F primer with fluorescent tag. We normally use FAM or HEX tags.

M13F-FAM primer: 5'-FAM-TGTAACGACGGCCAGT-3'

M13F-HEX primer: 5'-HEX-TGTAACGACGGCCAGT-3'

It is important to test primers using a panel of WT DNA from 4 to 8 fish of the same strain. A single robust peak (>1000 amplitude) is expected and detection of 2 peaks of similar amplitude in some or all of the samples indicates an in/del SNP. Sequence PCR product from these samples to identify the in/del and design new set of primers to avoid it.

**Please reference this paper when using protocol:**

Carrington, B., Varshney, G.K., Burgess, S.M. and Sood, R. (2015) CRISPR-STAT: an easy and reliable PCR-based method to evaluate target-specific sgRNA activity. *Nucleic Acids Res.*

CRISPR Calculator is online as a [supplemental file](#) and can be used to calculate sgRNA or multiplex guide injection volumes for the correct dose of *Cas9* and sgRNA.